



Europäisches Patentamt
European Patent Office
Office européen des brevets

Publication number:

**0 188 647
A1**

EUROPEAN PATENT APPLICATION

Application number: 85100715.3

Int. Cl.: **C 07 H 19/10, A 61 K 31/70**

Date of filing: 24.01.85

Date of publication of application: 30.07.88
Bulletin 88/31

Applicant: **NEOPHARMED S.p.A., Via Pordol, 18,
I-20021 Baranzate di Bollate Milano (IT)**

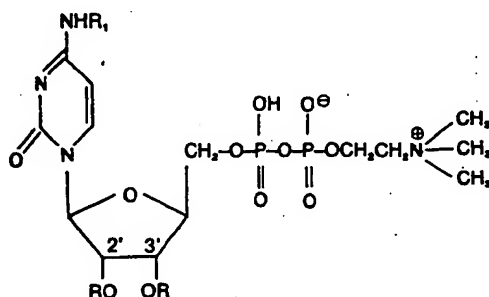
Inventor: **Zappla, Vincenzo, Via San Giacomo del
Capri 109, I-80100 Napoli (IT)**
Inventor: **De Rosa, Mario, Via Nicolardi 188,
I-80100 Napoli (IT)**

Designated Contracting States: **AT BE CH DE FR GB IT
LI LU NL SE**

Representative: **Böhling, Gerhard, Dipl.-Chem. et al,
Patentanwaltsbüro Tiedtke-Böhling-Kinne
Gruppe-Pelkmann-Grans-Struß/Bavariaring 4,
D-8000 München 2 (DE)**

Acylated derivatives of cytidine-diphosphate-choline, process for their preparation and their therapeutic use.

Derivatives of cytidine-diphosphate-choline of the following formula:



The compounds that respond to these requirements are mono-, di- and tri-acyl derivatives of CDP-choline that can be synthesized by acylation of CDP-choline with carboxylic acids using N,N'-carbonyldiimidazole.

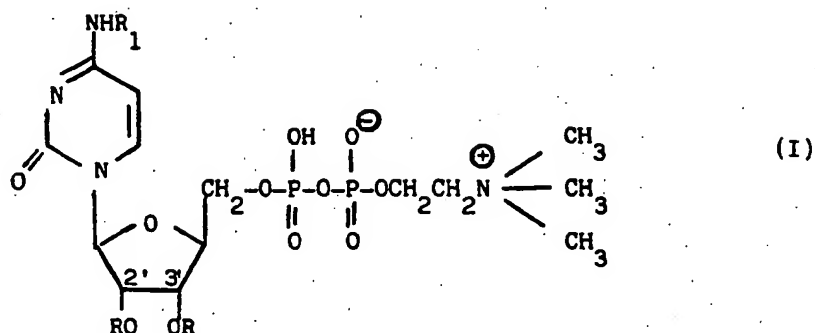
EP 0 188 647 A1

In which R represent an acyl radical chosen from the group comprised of saturated and unsaturated, linear or branched, monocarboxylic fatty acids having from 3-7 carbon atoms, and R₁ represents H or an acyl radical identical to R, show a therapeutic activity analogous to that of cytidine-diphosphate-choline and are suitable for oral administration in that they lead to a gradual release of the active principle in the gastrointestinal tract.

ACTORUM AG

BEST AVAILABLE COPY

This invention concerns novel derivatives of cytidine-diphosphate-choline having the general formula:



in which R represents an acyl radical chosen from the group comprised of monocarboxylic saturated and unsaturated, linear or branched, fatty acids having from 3 to 7 carbon atoms, and R_1 represents H or an acyl radical identical to R.

Cytidine-diphosphate-choline, referred to from now on as the abbreviation CDP-choline, represents the active form of choline and is a key intermediate in the biosynthesis of complex lipids. The importance of the cytidinic nucleotides in the formation of diester bonds in phospholipid molecules has been broadly documented in fact.

As shown in the literature, the lecithins and sphingomyelins are formed by means of a reaction catalysed by microsomal enzymes, in which the CDP-choline donates the phosphorylcholinic fragment to the D- α , β -diglyceride or to the N-acylsphingosine respectively.

Furthermore one should remember that CDP-choline acts as a P-choline donor to 1-alkyl, 1'-enyl-2-acyl-sn-glycerol, forming the plasmalogens.

CDP-choline is biosynthesized from P-choline and CTP, by means of the enzyme choline phosphate-cytidine transferase. This enzymic activity has been found in the particle free fraction of the cytoplasm and in the microsomal fraction. It is appropriate to underline, with reference to this, that the formation of CDP-choline

represents the slowest step and therefore the limitative one in the whole metabolic pathway. The cellular concentrations of this metabolite therefore have a critical importance in the regulation of the biosynthesis of phospholipids.

As a consequence of its biochemical roles, CDP-choline finds a use and pharmacological indication prevailing in a series of alterations in the central nervous system. In this organ, in fact, the structural and functional integrity of phospholipid membranes is particularly critical. The form of administration used up to now has been parenteral.

The pharmacological activity of the molecule has been demonstrated in various diseases such as the sequences of cerebral apoplexy, different types of cerebral ischaemia, Parkinson's disease, cranial fractures and their sequences.

This invention concerns the use of the acylated analogues of CDP-choline, as previously given in formula I, in therapy.

They are obtained by acylation at positions N_4 and/or 2', 3' of the molecule with the already defined carboxylic acids, in particular and preferably monocarboxylic acids, naturally present in mammals, of different chain lengths, linear or branched, saturated or unsaturated.

Esterification concerns some or all of the reactive positions on the molecule mentioned previously.

The acylated derivatives of CDP-choline are sufficiently stable in acid or alkaline environments so as not to be chemically broken down by the variations in pH associated with the journey through the gastro-intestinal tract.

The presence of strongly hydrophobic chains significantly modifies the molecular morphology, impeding, both at the gastric level and at the intestinal level, hydrolysis of the drug at the pyrophosphoric bond. This is possible only after removal of the acylic groups in sequence by the action of the gastric and intestinal acyl esterases and/or amidases and peptidases.

Drug absorption is conditioned by the hydrolysis of the acyl groups and therefore acylation of CDP-choline conditions the absorption of the cholinic and cytosinic components of the drug.

The acyl residues that are released can be considered as metabolites normally present in the organism with absolutely negligible toxicity levels.

The N_4 , 2', 3'-triacyl derivatives of CDP-choline administered orally act as retard forms of CDP-choline. The consequence of this process of slow release of CDP-choline in the organism is a more uniform and gradual distribution of the active principle.

As already indicated, according to this invention the CDP-choline derivatives have, in oral form, indications analogous to those for CDP-choline.

More simply, according to the invention, oral use of the CDP-choline derivatives is foreseen in table form, each containing from 200 to 1500 mg of the active principle, to be administered 2-3 times a day in the following illnesses:

- arteriosclerosis especially cerebral.
- short and long term treatment of cerebrovascular accidents.
- short and long term treatment of the consequences of a stroke.
- treatment of Parkinson's and Parkinson-like syndromes, in particular in the arteriosclerotic form.
- anti-depression treatment.
- treatment of cerebral traumatic coma.
- prevention and therapy of hyaline membrane disease (IRDS).
- therapy in acute and chronic hepatitis (viral hepatitis etc.).
- therapy and prevention of fatty liver in alcoholics.
- coadjuvant therapy in liver cirrhosis.

This inventions also concerns the preparation of the derivatives of CDP-choline of formula I. More specifically, the processes for the preparation of the derivatives (I) form another object of this invention, bound to the type of functionalisation or esterification desired. Therefore:

- 1) In N-monoacylation of CDP-choline at the aminic group of the carbon 4 of the pyrimidinic nucleus, the process, according to this invention, is characterized by the fact that the tetrabutylammonium salt of CDP-choline is reacted with an excess of imidazolidine of the desired carboxylic acid in an aprotic dipolar solvent (DMF, formamide or pyridine, of which DMF represents the best one), at a temperature of 50°C for 36 hours and in the presence of an acylation catalyst such as 4-(N,N-dimethylamino) pyridine.

The imidazolidine of the carboxylic acid is in turn prepared from the carboxylic acid by

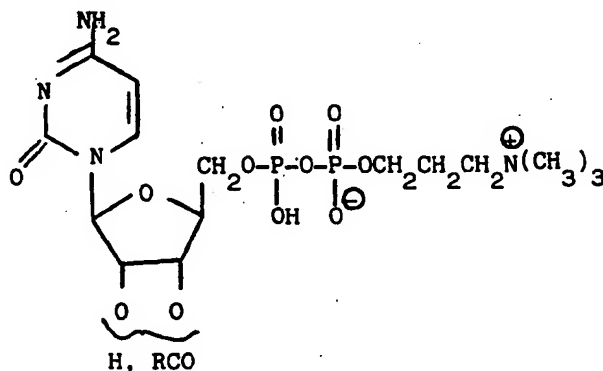
reaction with N,N'-carbonyldiimidazole in a solution of anhydrous DMF.

2) In the realisation of N⁴-acyl-2',3'-di-O-acyl-CDP-choline one can use the same method described in section 1 above, obviously using an excess of imidazolidine and, as a necessity, prolonging the time of the reaction to 96 hours, at a reaction temperature of 50°C.

3) Acylation with an aqueous mixture of poorly solvating compounds like THF, acetone, acetonitrile, the acylation takes place exclusively at the hydroxyl groups of ribose.

The best yields are obtained using mixtures H₂O/THF or H₂O/acetonitrile (1 : 4 v/v) as solvents. The acylation reaction never runs to completion and the best yields are obtained using a molar ratio of 2:1 between imidazolidine (as prepared separately in anhydrous THF) and CDP-choline.

Under these conditions monoacylation of CDP-choline takes exclusively place, indistinctly at one of the free hydroxyl groups at 2' or 3' of the ribose, giving 2'(3')-O-acyl-CDP-choline (Formula II).



(II)

The chromatographic purification of the reaction mixture on DOWEX AG 1:8 does not allow for resolution of the two isomers of CDP-choline acylated at 2' and 3' of the ribose respectively.

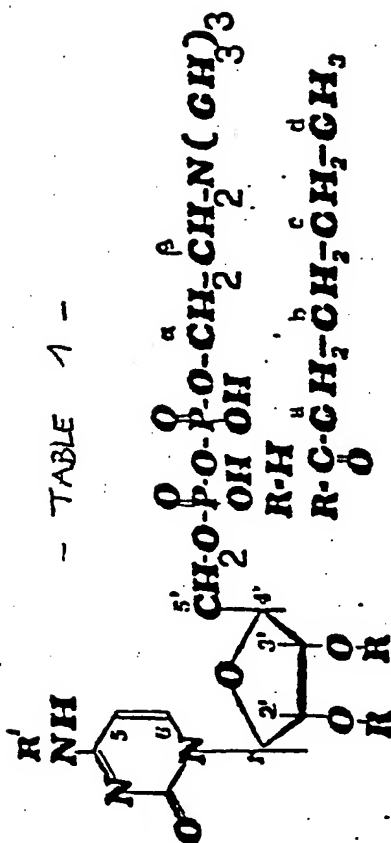
Therefore all the spectroscopic characterizations were carried out on the mixture of the two isomers that are stable in aqueous solution at pH 6.5 or lyophilized.

The identification of all the monoacyl and triacyl derivatives of CDP-choline is

essentially based on the ^1H -NMR and UV spectroscopic data.

The ^1H -NMR data concisely reported in Table 1 refer to the derivatives of valeric acid and are consistent with the structural assignments for each derivative (see Table 1).

TABLE 1 -



Assignment	6	5	1'	2'	3'	4'	5'	4	β	hch	a	T	b	c	d
Compound	Chemical shifts in δ														
1	8.16 d	6.27 d	5.88 d	4.41 - 4.20 c			3.69 s (wide)			3.22	/	/	/	/	/
2	"	"	6.12 c	5.38 - 5.46 c	4.68 - 4.39 c			"			2.49 c	1.62 q	1.33 se	0.69 t	
3	8.42 d	7.42 d	5.95 d	4.45 - 4.20 c			"			"	"	"	"	"	"
4	"	"	6.21 d	5.50 - 5.48 c	4.62 ABX	4.22-4.33 AUX	4.40 s (wide)	"			"	"	"	"	"

Table 1 - ^1H -NMR data for CDP-choline acylated derivatives. The spectra were obtained in $^2\text{H}_2\text{O}$ using a Bruker 500 MHz spectrometer. The chemical shifts are given in δ .

- given in O.
- 1) CDP-choline; 2) 2'(3')-O-valeryl-CDP-choline; 3) N⁴-valeryl-CDP-choline;
4) N⁴-valeryl-2',3'-di-O-valeryl-CDP-choline.
- se (sestet); q (quintet); t (triplet); d (duplet); s (singlet); c (complex signal)

The nuclear magnetic resonance spectrum in the case of 2'(3')-O-valeryl CDP-choline shows that only one acyl group has been introduced and is localised at the level of the 2' or 3' hydroxyl groups of ribose. One can in fact see a shift to lower fields on the part of the complex signal to 4,0-4,5, attributable to the H's on the hydroxylic group carrying carbons, whereas the proton's signals on the carbons 5 and 6 of the cytosinic nucleus are found at the same values of chemical shift that they have in CDP-choline thus excluding the possibility of an acylation of the aminic group at the carbon 4 of the aromatic system.

In the spectrum of N⁴-valeryl CDP-choline, however, the presence of a single unit of valeric acid per molecule is observed, specifically localised at the nitrogen in position 4 of the cytosinic system. In fact while the characteristic shift to lower fields of protons 5 and 6 of the chromophore is revealed, the ribose protons show resonance in the same fields as CDP-choline.

Finally, in the N⁴-valeryl-2', 3'-di-O-valeryl-CDP-choline spectrum, the integration of the protons of the acylating group shows the presence of three unit of valeric acid per molecule of CDP-choline. The total acylation of the molecule leads to a shift to low fields both of the aromatic protons and of the hydrogens of the ribose.

The examples that follow illustrate, without in any way limiting, the processes according to this invention.

EXAMPLE 1

Synthesis of 3'(2')-O-valeryl-CDP-choline

The chemical synthesis of CDP-choline acylated with valeric acid at the hydroxyl 2' or 3' of ribose, is carried out by condensation of CDP-choline with the imidazolide of valeric acid.

The latter, which is obtained by a reaction between valeric acid and N,N'-carbonyldiimidazole in a rigorously anhydrous environment, permits the acylation to be selectively carried out, in mild conditions and in an aqueous-organic environment, on one of the two ribose hydroxyl groups.

In a standard procedure, the preparation of the valeric imidazolide is carried out by reacting 1,3 g (8 mmoles) of carbonyldiimidazole with 500 mg (4.9 mmoles) of valeric

acid, in 5 mls of tetrahydrofurane, anhydried on molecular sieves and kept under anhydrous nitrogen. The reaction, carried out in nitrogen in very anhydrous conditions, is completed in about 10 minutes.

Like results are obtained if, while keeping the volume of solvent unaltered, double or triple quantities of the reactants are used or if the THF is substituted with acetonitrile or anhydrous acetone.

The acylation of CDP-choline is carried out in an aqueous-organic environment by mixing under stirring at room temperature 20 ml of a solution of 250 mM CDP-choline with 5 ml of the organic solution of imidazolide at different concentrations (1.6, 3.2, 4.8 M). In every case the reaction mixture initially appears like a stable white coloured emulsion; it becomes clear as the acylation of CDP-choline takes place. In about 4 hours the reaction reaches maximum yield. Working at 70°C the course of the reaction is much quicker (2h) but the yield of acylated product is not significantly modified. The 3'(2')-O-valeryl-CDP-choline can be isolated by evaporation of the solvent in a vacuum with a stream of nitrogen. The remaining aqueous solution is brought to pH 8.5 with 0.5N KOH and is then adsorbed onto a DOWEX 1X8 (formiate) column.

The elution is carried out first with H₂O and then with a linear gradient of formic acid from 0.00 to 0.02 M.

The 3'(2')-O-valeryl-CDP-choline has a very similar UV spectrum to that of CDP-choline, showing a maximum of 280 nm ($\epsilon = 12.8 \times 10^3$) in water.

The information that one can deduce from the analysis of the NMR spectrum of ¹³C fully confirms the structural identity of the molecule, excluding once again that the aminic group on the aromatic system has been involved in the acylation reaction.

In fact the cytosinic carbon signals, as in CDP-choline, have chemical shifts of 160.49 δ (C₂) 168.98 δ (C₄), 99.29 δ (C₅), and 144.28 δ (C₆) respectively. The ribose carbons however give rise to a good ten signals, five for each of the 2 isomers respectively acylated in positions 2' or 3', while the cholinic carbons are found at the same chemical shifts as in CDP-choline. On the other hand, at high fields one finds the aliphatic carbons of the valeric acid.

Structural analogues of CDP-choline, acylated at 2' or 3' with fatty acids from C₃ to C₇, saturated or unsaturated, linear or branched, can be obtained according to the

same reaction scheme given in example 1, substituting the valeric acid with the desired acid.

EXAMPLE 2

Synthesis of N⁴-valeryl-CDP-choline

0.2 mmoles of tetrabutylammonium hydroxide (1 ml 0.2 M) are added to 100 mg of CDP-choline, dissolved in 5 ml of H₂O, and then one proceeds to the lyophilisation of the sample. The tetrabutylammonium salt (TEBA) of CDP-choline is then dissolved in 4 ml of anhydrous DMF containing 20 mg of 4-dimethylaminopyridine.

2 mmoles of the imidazolid of valeric acid, obtained by reacting 204 mg of acid dissolved in 1 ml of anhydrous DMF with 324 mg of N,N'-carbonyl-diimidazole, are added to the resulting solution.

The reaction, stirred for 36 hours at 50°C, is interrupted by removing the solvent in a vacuum and the oleaginous residue obtained is triturated three times with ethyl acetate.

The reaction mixture is purified by chromatography on silica, by elution with H₂O in CH₃OH in a linear gradient from 0 to 20% v/v. The N⁴-valeryl-CDP-choline is obtained with a yield of 50-60% and N⁴-valeryl-2',3'-di-O-valeryl-CDP-choline, N⁴-acylated with fatty acids from C₃ to C₇, saturated or unsaturated, linear or branched, can be obtained according to the method given in this example, substituting the valeric acid with the acid desired.

EXAMPLE 3

Synthesis of N⁴-valeryl-2',3'-di-O-valeryl-CDP-choline

One proceeds as described in the former example up to the preparation of the solution of the TEBA salt of CDP-choline in DMF, containing 4-(N,N-dimethylamine) pyridine.

At time nought and after 48 hours, 2 mmoles of the imidazolid of valeric acid, obtained by reacting 204 mg of the acid dissolved in 1 ml of anhydrous DMF with 324 mg of N,N'-carbonyl-diimidazole, are added to this solution. The reaction mixture is stirred for 96 hours at 50°C, and the reaction is interrupted by removing the solvent in a vacuum and triturating the oleaginous residue obtained three times with ethyl acetate.

The triacylate (105 mg) is isolated pure by chromatography on a silica column by

elution with H_2O in CH_3OH in a linear gradient from nought to 20% v/v.

Structural analogues of CDP-choline, triacylated with fatty acids from C_3 to C_7 , saturated or unsaturated, linear or branched, can be obtained according to the same method given in example 3, substituting the valeric acid with the acid desired.

The pharmacokinetic characteristics of N^4 -valeryl-2',3'-di-O-valeryl-CDP-choline have been defined in rats by oral administration of the product with a double label of 3H at 5' on the cytosinic nucleus and ^{14}C on the cholinic methyls. The required data refer to the distribution of radioactivity in the animal and also to structural identity of the radioactive molecular species present in some of the more significant biological samples (liver, brain, faeces, gastric and intestinal contents).

A precise analysis of the role played by the functionalisation in the absorption process and metabolism of the triacylated drug has imposed a comparison of the obtained data with those of a control experiment, in which rats were given orally equimolecular doses of (5- 3H ; methyl- ^{14}C) CDP-choline. The characterization of the labelled metabolites present in the liver, brain, faeces, gastric and intestinal contents of the animals treated orally with pharmacological doses (20 μ moles/kg) of (5- 3H ; methyl- ^{14}C)-CDP-choline or of N^4 -valeryl-2',3'-di-O-valeryl-(5- 3H ; methyl- ^{14}C)-CDP-choline leads to their preventive extraction from the tissues.

The scheme of extraction consents the recovery of three different classes of labelled metabolites:

- 1) Hydrosoluble metabolites, subsequently analysed by HPLC.
- 2) Metabolites with high molecular weights, proteins and nucleic acids, recovered by thermal denaturation in a hydroalcoholic environment.
- 3) Lipids, subsequently purified by chromatography on silica.

The experimental results show that N^4 -valeryl-2',3'-di-O-valeryl CDP-choline is an interesting oral form of slow release of CDP-choline.

The global analysis of the metabolic picture, as compared to that found in the experiment with CDP-choline, substantially shows a better and more gradual distribution of the radioactivity in the organism. The metabolic pictures of the organs examined (liver and brain) were substantially equal and acylated cytosinic metabolites were not found. This indicates that the valeric acid chains are quantitatively removed

before the cytosinic component is used which does not appear to be significantly compromised in comparison with the control experiment.

The bioavailability in terms of recovery of the structural components of the drug in 24 hours is very similar for CDP-choline and its triacylated analogue, the faecal and urinary excretions being quite comparable.

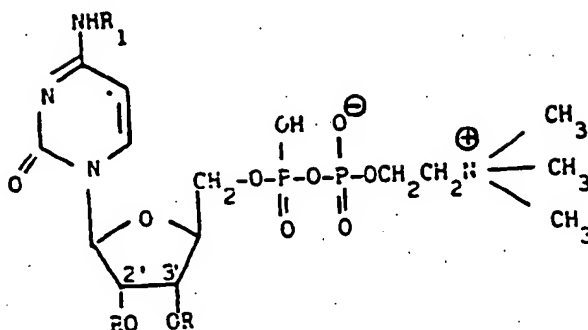
The radioactive levels and the structural identity of the labelled species present at gastric and intestinal level, clearly show the notable consistency of the protective effect exercised by the valeric acid residues as regards the pyrophosphatases, intestinal especially, and the indirect control operated on the speed of absorption.

In fact, while after 30 minutes in the control experiment at intestinal level not more than 5% of the radioactivity present in the dose and only 0.2% of the double labelled CDP-choline administered is found, on the other hand in the case of trivaleryl-CDP-choline 29% of the variably acylated double labelled molecule is still found after an hour and a total radioactivity equal to 30% of that present in the administered dose is detected.

To confirm this datum, in the plasmatic component and in the organs, the maximum uptake is definitely moved to longer periods of time (5 hours) with trivaleryl-CDP-choline.

CLAIMS

1. Derivatives of CDP-choline having the formula:



in which R represents an acyl radical chosen from the group comprised of saturated or unsaturated, linear or branched monocarboxylic fatty acids having from 3 to 7 carbon atoms, and R_1 represents H, or an acyl radical identical to R, or different when R_1 = acyl radical and R = H.

2. A process for the preparation of the O-monoacylated derivatives of CDP-choline of formula I, wherein R_1 =H, characterized by reacting CDP-choline with the acylimidazole (imidazolide) of the desired carboxylic acid in an aqueous mixture of a poorly solvating compound.
3. A process according to claim 2, characterized in that the carboxylic acid is a saturated or unsaturated, linear or branched monocarboxylic fatty acid containing 3 to 7 carbon atoms.
4. A process according to claim 2, characterized in that said poorly solvating compound is selected among tetrahydrofuran, acetone and acetonitrile.
5. A process according to claim 4, characterized in that the reaction medium is a mixture 1:4 v/v of water and the poorly solvating compound.
6. A process according to claim 2, characterized in that the molar ratio between imidazolide and CDP-choline is 2:1.
7. A process for the preparation of N⁴-monoacylated derivatives of formula I in which R_1 =acylic radical and R = H, characterized by the fact the tetrabutylammonium salt of CDP-choline, in a homogeneous solution of an aprotic dipolar solvent and in the presence of an acylation catalyst, is reacted with the imidazolide of the carboxylic

acid to be introduced at N⁴.

8. A process according to claim 7, characterized in that said solvent is selected among formamide, pyridine and dimethylformamide.
9. A process according to claim 7, characterized in that said acylation catalyst is 4-N,N-dimethylaminopyridine.
10. A process according to claim 7, characterized in that the reaction is carried out with an excess of imidazolidine.
11. A process according to claim 7, characterized in that the reaction is carried out at 50°C and for 36 hours.
12. A process for the preparation of N⁴-acyl-2',3'-di-O-acyl derivatives of CDP-choline of formula I, characterized in that the reaction is carried out according to claim 7, with an excess of imidazolidine, for a time of 96 hours and at a temperature of 50°C.
13. Pharmaceutical composition characterized by containing as the active ingredient, a derivative of CDP-choline, according to claim 1, together with the usual excipients and carriers.
14. Pharmacological composition according to claim 13, characterized by the fact of being in a form suitable for oral administration.
15. Pharmaceutical composition according to claim 13, useful for the treatment of cerebral apoplexy, cerebral ischaemia, Parkinson's disease, cranial traumatology.
16. Pharmaceutical composition according to claims 13 and 14, characterized by containing 200 to 1500 mg of the active principle, as a unitary dosage.



European Patent
Office

EUROPEAN SEARCH REPORT

0188647

Application number

EP 85 10 0715

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	"The Merck Index; An Encyclopedia of Chemicals and Drugs", 9th edition, 1976, page 299, Merck & Co., Inc., Rahway, N.J., US; * Page 299, abstract no. 2300, "Citicoline" *	1,13	C 07 H 19/10 A 61 K 31/70
A	--- CHEMICAL ABSTRACTS, vol. 100, no. 25, 18th June 1984, page 645, no. 210323s, Columbus, Ohio, US; M. MARIAN: "Acetyl derivatives of nucleoside 5'-triphosphates" & MICROCHEM. J. 1984, 29(2), 219-27 * Abstract * -----	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 07 H 19/00 A 61 K 31/00
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 30-08-1985	Examiner VERHULST W.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO Form 1503, 03.82